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Olfactory experience primes the heat shock transcription factor HSF-1 to enhance the expression of molecular chaperones in *C. elegans*

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Abstract

Learning, a process by which animals modify their behavior as a result of experience, enables organisms to synthesize information from their surroundings to acquire resources and avoid danger. We showed that a previous encounter with only the odor of pathogenic bacteria prepared *Caenorhabditis elegans* to survive exposure to the pathogen by increasing the heat shock factor 1 (HSF-1)-dependent expression of genes encoding molecular chaperones. Experience-mediated enhancement of chaperone gene expression required serotonin, which primed HSF-1 to enhance the expression of molecular chaperone genes by promoting its localization to RNA polymerase II-enriched nuclear loci, even before transcription occurred. However, HSF-1-dependent chaperone gene expression was stimulated only if and when animals encountered the pathogen. Thus, learning equips *C. elegans* to better survive environmental dangers by preemptively and specifically initiating transcriptional mechanisms throughout the whole organism that prepare the animal to respond rapidly to proteotoxic agents. These studies provide one plausible basis for the protective role of environmental enrichment in disease.

INTRODUCTION

The ability to accurately predict danger and implement appropriate protective responses is critical for survival. Many animals have neuronal circuits that detect unfavorable conditions and initiate an avoidance response. In addition, all cells have conserved mechanisms to repair and protect macromolecules from damage that occurs under adverse conditions. One such mechanism present in all cells to protect against protein damage is the heat shock response (1–4). The heat shock response is mediated by the transcription factor heat shock factor 1 (HSF1), which, in response to a variety of stressors, induces the expression of cytoprotective heat shock proteins (HSPs), which are molecular chaperones that maintain

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SUPPLEMENTARY MATERIALS

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protein stability and help degrade proteins that misfold and aggregate under stressful conditions (1–4). HSF1 activity is essential for all organisms to adapt to changing environments. Because the heat shock response has been characterized predominantly in mammalian cells in culture and in unicellular organisms such as yeast, the activation of HSF1 has been considered an autonomous response of cells to protein damage caused by stressors (1–3). However, emerging evidence has shown that within a metazoan such as the nematode *Caenorhabditis elegans* HSF1 (HSF-1) and the cellular response to protein damage are not autonomously controlled by individual cells, but instead are under the regulation of the animals' nervous system (5–11). The biological role for such systemic—rather than cell-autonomous—regulation is unclear. We discovered that one mechanism by which the activity of *C. elegans* HSF-1 is regulated is through the neurosensory release of the bioamine serotonin [5-hydroxytryptamine (5-HT)] (7). In vertebrates and invertebrates, serotonergic systems play a central role in neurophysiological processes underlying learning and memory, allowing animals to learn about threats in their environment and form memories that can be later recalled to modify behavior (12–23). Therefore, we asked whether control by the serotonergic-based learning circuitry allowed *C. elegans* to modulate HSF-1 activity in response to prior experience, so as to better combat threats in its environment.

Here, we show that, in *C. elegans*, olfactory experience of specific odorants released by the toxic bacterium *Pseudomonas aeruginosa* PA14 primed HSF-1–dependent transcription of cytoprotective *hsp* genes, such that the expression of these genes was enhanced if and when the animals subsequently encountered the pathogen. This priming required 5-HT and appeared to be a consequence of the preemptive mobilization of HSF-1 to the vicinity of RNA polymerase II (pol II) in nuclei throughout the animal. Animals that cannot synthesize 5-HT were deficient in relocating HSF-1 in response to olfactory stimuli and did not show this learning-dependent enhancement of *hsp* expression. Olfactory priming of HSF-1 was protective, allowing animals that had previously experienced only the smell of *P. aeruginosa* to better respond to a subsequent exposure to the pathogen itself. We conclude that neuronal control over the HSF-1–mediated defense mechanism of cells allows learning and memory to elicit anticipatory changes that promote the ability of cells to respond to stress, thus facilitating survival.

RESULTS

Olfactory exposure to odorants produced by the toxic bacterium *P. aeruginosa* PA14 accelerates the pathogen avoidance response of *C. elegans*

To test whether prior experience primes animals to activate HSF-1, we set up a paradigm to train *C. elegans* to avoid sensory stimuli that are predictive of damage and asked whether pre-exposure to such stimuli affected HSF-1 activity. To do this, we exploited previous findings that *C. elegans* have an innate aversion to specific pathogens and display experience-dependent plasticity to avoid ingesting pathogenic bacteria such as *P. aeruginosa* strain PA14 (15, 24). Although *C. elegans* are typically attracted to any novel bacterium, be it pathogenic bacteria such as PA14 or nonpathogenic *Escherichia coli* strains (25, 26), animals previously exposed to a lawn of pathogenic PA14 will avoid PA14 lawns upon

subsequent exposure. This learned avoidance behavior requires the olfactory nervous system and 5-HT (15, 24, 27, 28). We used this information to train animals to avoid PA14 using odor alone, thereby circumventing any physical damage that could be inflicted by actual exposure to the pathogen (Fig. 1A and fig. S1A). We then asked whether olfactory training on the odor of this toxic bacterium could enhance the animals' avoidance behavior if they were to subsequently encounter the pathogen. Animals were trained by exposing them to the odor of PA14 cultures for 30 min. Controls were mock-trained by exposure to the odor of the standard nonpathogenic *E. coli* OP50 strain on which the animals are typically raised. To assess whether olfactory pre-exposure was sufficient to elicit learned avoidance behavior, trained and mock-trained animals were then immediately given a choice between PA14 lawns and OP50 lawns. Behavioral preference was quantified by calculating a choice index (CI) for PA14, wherein a CI of 1.0 indicates maximal preference and a CI of -1.0 indicates maximal aversion (Fig. 1A). Because of the variability inherent to behavioral assays, all avoidance assays were conducted and are represented as pairwise comparisons between control and experimental populations of *C. elegans* evaluated in parallel. As previously reported (15), when faced with a choice between OP50 or PA14, naïve animals initially preferred the novel bacterium and accumulated on PA14 within 5 min (fig. S1B). However, after 45 min, the animals began to avoid PA14, and by 4 hours, 80% of the animals had left the lawn of PA14 and moved to the lawn of OP50 (fig. S1B). Animals pre-exposed to the OP50 odor (mock-trained, control animals) behaved like naïve animals and also initially accumulated on PA14 and then began to leave the lawn by 1 hour (Fig. 1B). In contrast, animals pre-exposed to the odor of PA14 avoided the PA14 lawn significantly earlier and left within the first 5 min (Fig. 1B). The avoidance of PA14 after pre-exposure to PA14 odorants appeared to reflect an innate response of the animals to PA14. It was also not a simple consequence of adaptation to the smell. This was inferred from the behavior of animals exposed for similar durations to the odor of another novel, but nonpathogenic, bacterium, *E. coli* HT115. In this case, animals did not avoid HT115 when given a choice between HT115 and OP50 but remained on HT115 throughout the analysis (fig. S1C). This enhanced avoidance response was also specific to the pathogen in that animals responded to the pathogen whose odorants they had previously experienced. Pre-exposure of animals to the odor of PA14 did not trigger avoidance of another known *C. elegans* pathogen, *Serratia marcescens* strain DB11. Animals pre-exposed to PA14 or OP50 odorants behaved like naïve animals and remained on DB11 throughout the analysis (fig. S1D). These data together point to the existence of sophisticated mechanisms by which *C. elegans* discriminate between bacteria in their environment and show that prior exposure to odorants generated by a pathogen such as *P. aeruginosa* can induce *C. elegans* to accelerate their avoidance of that specific pathogen upon subsequent encounter.

The HSF-1–dependent expression of HSP genes is enhanced by prior olfactory exposure to PA14 odorants

Exposure to PA14 is toxic to *C. elegans*, causing increased protein damage (29, 30) and ultimately death (31, 32). Consistent with this, survival on PA14 was dependent on HSF-1. Knocking down *hsf-1* by RNA interference using standard methods for feeding double-stranded RNA (dsRNA) to *C. elegans* accelerated death upon PA14 exposure (Fig. 1C, table S1, and fig. S4A). To assess whether training on PA14 odorants modulated the HSF-1–

mediated transcriptional response, we placed animals exposed to OP50 odorants or PA14 odorants on PA14 lawns that covered the entire surface area that was available to the animals. Under these conditions, HSF-1 was activated: All animals placed on PA14 lawns for only 10 min increased HSF-1–dependent expression of one or both of the identical inducible *hsp70* genes (F44E5.4 and F44E5.5) and the small HSP genes (*hsp-16.2* and *hsp-16.41*), as measured by qRT-PCR (Fig. 1, D to F, and fig. S1E). Pre-exposure to the odor of PA14, however, enhanced this HSF-1–dependent transcriptional response (Fig. 1, D to F, and table S2). The amounts of all these chaperone mRNAs were about twofold higher in animals that were pre-exposed to the odor of PA14 compared to control animals pre-exposed to the smell of OP50 (Fig. 1, D to F, and table S2). This suggested that HSF-1–mediated gene expression could be enhanced by prior experience of signals that were predictive of danger. Pre-exposure to the odor of PA14 did not in itself induce expression of chaperone genes; animals exposed to the odor of PA14 had low basal chaperone expression similar to control animals (Fig. 1, D to F, and table S2).

P. aeruginosa releases several molecules that alter the behavior of other organisms. The “grape-like” odorant 2-aminoacetophenone (2AA) is one such compound synthesized relatively early in the growth cycle and is enriched when *P. aeruginosa* infects animal tissues, such as the wounds of human burn victims or the lungs of patients with cystic fibrosis (33, 34). 2AA is responsible for the attractive behavior of *Drosophila melanogaster* toward the pathogen (33, 35) as well as the aversive behavior of vertebrate species such as birds and mice from *Pseudomonas* (36, 37). We tested whether this compound was, at least in part, responsible for the learned enhanced aversion of *C. elegans* to PA14. Pre-exposure to 2AA mimicked the results observed in our choice assay (Fig. 2A), but 2AA did not in itself elicit an avoidance response (fig. S2A). Animals that were pre-exposed to the smell of 2AA avoided PA14 lawns by 15 min compared to mock-trained, control animals exposed to water as the odorant, who only began to avoid PA14 lawns by 45 min (Fig. 2A). Pre-exposure to 2AA odorant also enhanced the expression of *hsp70* when animals were subsequently exposed to PA14 lawns, but the 2AA odorant itself did not induce *hsp70* expression (Fig. 2B and table S2). Moreover, the avoidance of 2AA did not appear to be due to its potential toxicity, and prolonged 2AA exposure had no effect on the life span of animals, be it administered as an odor alone (Fig. 2C and table S3) or mixed into OP50 for direct contact and ingestion (Fig. 2D and table S3). The enhancement of PA14-avoidance behavior by 2AA also appeared to be somewhat specific, because pre-exposure to another volatile semiochemical secreted by *Pseudomonas*, *N*-3-oxododecanoyl homoserine lactone (38), did not affect subsequent avoidance of PA14 lawns or enhance *hsp* gene expression (fig. S2, B and C, and table S2). Consistent with a role in signaling a potential threat, pre-exposure to 2AA appeared to facilitate a mechanism by which animals activated HSF-1 only if they subsequently encountered PA14. When *C. elegans* that were pre-exposed to 2AA odorant encountered an OP50 lawn instead of a PA14 lawn, they did not activate HSF-1–dependent *hsp* gene expression (Fig. 2B and table S2). However, if animals did encounter PA14, pre-exposure to PA14 odorants conferred a consistent and significant survival advantage: 63% of the animals pre-exposed to PA14 odor survived after 18 hours of PA14 exposure, compared to 46% of control animals exposed to OP50 odor (Fig. 2E and table S4). The protection conferred by pre-exposure to PA14 was also stressor-specific, enhancing survival on PA14

but not upon prolonged heat stress (fig. S2D). These data together suggest that the prior experience of PA14 odor, mimicked in large part by the odorant 2AA, enhanced the organism's ability to survive, not only by hastening the avoidance behavior of the animal from the pathogen but also by enhancing the expression of cytoprotective HSF-1 transcriptional targets upon encounter with the pathogen itself. 2AA itself was not toxic or aversive but instead appeared to convey specific information regarding the bacterial environment that prepared *C. elegans* for survival on the pathogen and conferred in some unknown way a degree of specificity to the HSF-1 transcriptional response.

Serotonin is required for olfactory training to enhance HSF-1–dependent *hsp* gene expression upon exposure to PA14

In *C. elegans* and other organisms, the neuromodulator 5-HT mediates learning (15, 21, 22, 26, 39–42). We therefore tested whether the enhanced behavioral and transcriptional response to PA14 that occurred after the pre-exposure of *C. elegans* to PA14-derived odors required 5-HT. Compared to the 5 min needed for wild-type animals trained on PA14 odor to avoid PA14 lawns, *tph-1* mutant animals that lack functional tryptophan hydroxylase (43), the rate-limiting enzyme for 5-HT synthesis, took 1 hour to avoid PA14 after pre-exposure to the odor of PA14 (Fig. 3A and fig. S3A). This delay in avoidance was due to the lack of 5-HT, because even transient incubation with exogenous 5-HT before training, which causes 5-HT to be loaded into serotonergic neurons (fig. S3B) (42), rescued the deficiency in the learned response of *tph-1* mutant animals. *tph-1* mutant animals treated with 5-HT and trained on PA14 odor acted like wild-type animals trained on PA14 odor and avoided PA14 lawns within 5 min (Fig. 3B and fig. S3C, compare to Fig. 1A). 5-HT was not required for the detection or the attractiveness of PA14, because *tph-1* mutants were also first attracted to, then subsequently avoided PA14 lawns. However, for reasons we do not understand, but which may be related to the role of 5-HT in providing both excitatory and inhibitory inputs to modulate olfactory behavior (44, 45), control, mock-trained *tph-1* animals lacking 5-HT avoided PA14 lawns earlier than wild-type control animals (fig. S3A, compare to Fig. 1A). This aberrant behavior of mock-trained *tph-1* animals was reversed with exogenous 5-HT treatment: 5-HT–treated *tph-1* animals mock-trained on OP50 odor acted more like wild-type animals exposed to OP50 odor and avoided PA14 lawns later, by 45 min (fig. S3, C and D). However, this rescue was variable and did not reach significance.

Consistent with the behavioral response, *tph-1* mutant animals were deficient in the enhanced HSF-1–dependent transcriptional response elicited by pre-exposure to PA14 odor (Fig. 3C and table S2), although 5-HT was not required for HSF-1 activation on PA14 per se. We inferred this from the observation that *tph-1* mutant animals did induce *hsp70* (F44E5.4/F44E5.5) expression when exposed to PA14 lawns as assessed by qRT-PCR (Fig. 3C and table S2). However, *tph-1* mutant animals pre-exposed to OP50 or PA14 odors both expressed similar amounts of *hsp70* (F44E5.4/F44E5.5) mRNA upon subsequent encounter with PA14 lawns, and there was no increase in *hsp70* expression on the basis of prior olfactory experience (Fig. 3C and table S2). We tested whether treatment with exogenous 5-HT could also reverse this defect. However, consistent with what we had previously observed upon optogenetic activation of serotonergic neurons (7), exposure to exogenous 5-HT induced the expression of *hsp70* (F44E5.4/F44E5.5) mRNA even without pre-exposure

to PA14 odor (Fig. 3D). Although these data confirm the role of 5-HT in triggering HSF-1 activity, such data suggest that the fine control over HSF-1-mediated gene expression that occurs in the animal in response to physiological stimuli may be due to a more stringent regulation of 5-HT release and availability.

Because 5-HT is synthesized only in neurons in *C. elegans* whereas 5-HT receptors are expressed in multiple tissues (39, 43), we tested whether 5-HT-dependent HSF-1 activation was restricted to neurons or whether it occurred throughout the animal. To do this, we used single-molecule fluorescence in situ hybridization (smFISH) to detect *hsp70* (F44E5.4/F44E5.5) mRNA across the entire organism. smFISH indicated that exposure to PA14 induced F44E5.4/F44E5.5 mRNA in all tissue types including neurons, the intestine, and the germ line, and mRNA expression was enhanced in all these tissues in wild-type animals trained on PA14 odor (Fig. 4, A to F). Consistent with the whole-animal qRT-PCR data, *tph-1* mutant animals also induced *hsp70* (F44E5.4/F44E5.5) mRNA when exposed to PA14, but the induction of mRNA in *tph-1* mutant animals remained the same irrespective of prior olfactory training and was also similar to that in control, wild-type animals mock-trained with OP50 (Fig. 4, A to F). Together, these studies showed that both enhanced avoidance behavior and enhanced HSF-1-dependent chaperone gene expression were mediated by the 5-HT learning circuitry.

Olfactory training and serotonin cause HSF-1 to localize to nuclear bodies and prime HSF-1-dependent gene expression

How might olfactory learning enhance HSF-1 transcriptional activity? To answer this, we examined whether olfactory training modified any of the steps known to accompany HSF-1 activation. HSF1-dependent transcription of *hsp* genes is a multistep process that varies somewhat between species (1, 4, 46–51). In mammalian cells, HSF1-dependent *hsp* expression involves the conversion of HSF1 monomers to trimers, increased phosphorylation and other posttranslational modifications, acquisition of competence to bind heat shock elements (HSEs) in the promoters of *hsp* genes, and recruitment of HSF1 to HSEs in a manner that depends on the chromatin landscape and transcriptional machinery. We characterized these steps of HSF-1 activation in *C. elegans* (figs. S4 and S5, A and B). Consistent with its essential role in development (52), *C. elegans* HSF-1, as detected by an antibody specific for endogenous *C. elegans* HSF-1 (fig. S4, A and B) and by the localization of green fluorescent protein (GFP)-tagged HSF-1, is constitutively present in nuclei (fig. S4C) (7, 51, 53), is likely phosphorylated (fig. S4D), and appears to be present as a trimer (fig. S4E) even at ambient temperatures. Electrophoretic mobility shift assays (EMSAs) indicated that in accordance with trimerization at ambient temperatures, *C. elegans* HSF-1 can bind DNA containing canonical *C. elegans* HSEs in vitro (fig. S5, A and B). The ability of *C. elegans* HSF-1 to bind HSE-containing DNA in vitro does not change with stress-induced transcriptional activation (fig. S5, A and B). However, in agreement with the lack of expression of inducible *hsp* genes at ambient temperatures in the absence of stress, *C. elegans* HSF-1 did not constitutively bind the *hsp70* promoter region in vivo as assayed by chromatin immunoprecipitation and qPCR (ChIP-qPCR) (fig. S5C). Instead, HSF-1 binding to the *hsp70* (F44E5.4/F44E5.5) promoter in vivo required a stressor such as

heat shock, which caused transcriptional activation and an about fivefold enrichment of HSF-1 at the *hsp70* (F44E5.4/F44E5.5) locus (fig. S5C).

Olfactory pre-exposure to PA14 odor or the PA14 odorant 2AA did not enhance the ability of HSF-1 to bind DNA in vitro as determined by EMSA (fig. S5, A and B), nor did it cause HSF-1 to bind *hsp70* promoter regions in vivo as indicated by ChIP-qPCR (fig. S5C). HSF-1 appeared to be phosphorylated upon exposure of animals to PA14 lawns, as visualized by its retarded mobility in SDS–polyacrylamide gel electrophoresis (PAGE); however, pre-exposure to 2AA did not induce this posttranslational change, and HSF-1 in both control (water trained) and 2AA-trained animals appeared identical by SDS-PAGE analysis (fig. S5D). Exposure to 2AA odor or PA14 odor alone, however, without exposing the animals to a lawn of PA14, caused a significant fraction of HSF-1 to relocalize to punctate nuclear bodies (Fig. 5, A and B). This was similar to changes in HSF-1 localization that occur when HSF-1 is actively transcribing *hsp* genes in response to stressors such as increased temperatures (fig. S4C) (7, 51, 53) and PA14 lawns (Fig. 5A). Therefore, the formation of nuclear bodies upon 2AA exposure alone was surprising, because exposure to 2AA odor did not induce the transcription of *hsp* genes. The number of nuclei containing HSF-1 nuclear bodies after exposure of animals to 2AA averaged 8.7%, ranging from 3 to 42% among the germline nuclei, where it was easiest to visualize HSF-1 and was visible in 71% of animals scored (Fig. 5, A and B). In comparison, only an average of 2.2% of germline nuclei ranging between 0 and 10% (Fig. 5, A and B) of control animals exposed to the odor of water showed any evidence of HSF-1 nuclear bodies. The relocalization of HSF-1 into nuclear bodies was reversible, and the numbers of nuclei with HSF-1 nuclear bodies in animals exposed to PA14 odorants diminished to control values (3.0%) after 30-min recovery on OP50 and did not differ from that in control water-exposed animals (3.2%; Fig. 5, A and B).

The HSF-1–mediated transcriptional memory of pre-exposure to PA14 odors that resulted in enhanced HSF-1–dependent *hsp* gene expression correlated with the presence of HSF-1 nuclear bodies (Fig. 5, A and C). Whereas animals exposed to the PA14 odorant showed the presence of HSF-1 nuclear bodies and displayed enhanced expression of *hsp* genes when placed on PA14 lawns, animals that were allowed to recover for 30 min on innocuous OP50 lawns after being trained on PA14 odorants no longer displayed enhanced *hsp* gene expression when placed on PA14 lawns (Fig. 5, A and C; compare with Figs. 1D and 2B). In further support of the role of HSF-1 nuclear bodies in the learning-dependent enhancement of HSF-1 transcription, *tph-1* mutant animals that lacked 5-HT and were deficient in olfactory experience–mediated increase in *hsp* gene expression also had markedly fewer HSF-1 nuclear bodies upon olfactory training (Fig. 5, D and E). Together, these data indicate that the priming of HSF-1 upon olfactory exposure to PA14 odorants, which resulted in an enhancement of *hsp* gene expression upon encounter with PA14 lawns, occurred through the mobilization of HSF-1 to nuclear bodies throughout cells of the animal.

HSF-1 nuclear bodies colocalize with RNA pol II

Transcription does not occur homogeneously throughout the nucleus but rather occurs at specialized, discrete sites (54). We hypothesized that because animals that were trained on PA14-derived odors did not induce *hsp70* gene expression unless they encountered PA14

lawns, olfactory signaling may facilitate the association of HSF-1 with the transcriptional machinery, thus preparing the animal for a transcriptional response if an actual encounter with the threat (PA14) were to occur. We therefore investigated whether the location of HSF-1 nuclear bodies corresponded to known sites of enriched transcriptional activity. In *C. elegans*, the *hsp-16.2* promoter localizes to the nuclear pore complex (NPC) after heat shock (54, 55). However, although we occasionally observed HSF-1 nuclear bodies in the vicinity of NPCs in germline nuclei, they did not colocalize with NPCs under any of our conditions (Fig. 6, A to C). On the other hand, more than half of the HSF-1 nuclear bodies (0.2 of 0.3 nuclear bodies per nucleus) that were induced by olfactory exposure to 2AA colocalized with RNA pol II (Fig. 6, D to F). The number of HSF-1 nuclear bodies that colocalized with pol II remained the same even when HSF-1 was actively involved in pol II-dependent transcription of *hsp* genes, such as upon heat shock or when animals were exposed to PA14 lawns (Fig. 6, D to F). In comparison, few of the rare HSF-1 nuclear bodies visible in control animals colocalized with pol II (Fig. 6, D to F). Consistent with previous reports (46, 56–58), pol II appeared to cluster in discrete nuclear regions even before 2AA exposure or heat shock (Fig. 6, D to F). The formation of HSF-1 nuclear bodies, however, did not appear to require pol II: RNA interference (RNAi)-induced knockdown of the large subunit of RNA pol II (AMA-1) substantially decreased the amounts of pol II protein in oocyte nuclei (fig. S6A) but did not interfere with the heat shock-induced formation of HSF-1 nuclear bodies in oocytes (fig. S6B). We conclude from these studies that olfactory training with PA14 odorants primed HSF-1 for promoting transcription by preemptively concentrating it at nuclear loci in close proximity to RNA pol II. Although we do not yet understand the nature of these nuclear foci where HSF-1 and pol II were concentrated or the intracellular mechanisms by which this occurred, together, these data suggest that the ability of the serotonin-based learning circuitry to induce the colocalization of HSF-1 with pol II in nuclei throughout the animal in response to only the odor of the pathogen could result in an enhanced transcriptional response upon actual exposure to the pathogen (46, 47, 55–58).

HSF-1 is required for the learned avoidance behavior of *C. elegans* toward PA14

Not only was HSF-1 activity enhanced by aversive olfactory stimuli, HSF-1 appeared to be required for the behavioral avoidance of PA14. This was evidenced in choice assays where animals subject to RNAi-induced knockdown of *hsf-1* were pre-exposed to the odor of PA14 and assessed for their behavioral avoidance of PA14 lawns (Fig. 7A and fig. S7A). All animals for these experiments were grown on a strain of dsRNA-expressing bacteria used for inducing the knockdown of genes in *C. elegans* rather than on OP50. For this reason, the odor of control RNAi-expressing bacteria was used as the control to train animals, instead of the odor of OP50, and animals were given a choice between lawns of control RNAi-expressing bacteria and PA14. Decreasing the amounts of *hsf-1* mRNA and protein abrogated the behavioral plasticity observed upon exposure to PA14, and animals deficient for *hsf-1* remained equally distributed between the PA14 and lawns of control RNAi-expressing bacteria, indicating a deficiency in their avoidance of PA14 (Fig. 7A and fig. S7A). Loss of *hsf-1* slightly retarded motility, causing a delay of ~102 s for *hsf-1* RNAi-treated animals to traverse the 1-inch distance between the PA14 and OP50 lawns as compared to wild-type animals (fig. S7B). However, this slight decrease in motility rates could not account for the lack of avoidance behavior of *hsf-1* RNAi-treated animals,

because they did not avoid PA14 even by 4 hours after the start of the choice assay. By this time, all wild-type animals raised on control RNAi, whether trained on PA14 odor or the odor of control RNAi-expressing bacteria, had left the PA14 lawns (Fig. 7A). It therefore appeared that 5-HT signaling was integrating olfactory information and HSF-1 activation to flag a sensory stimulus as a threat, providing a basis for the coupling of the enhanced behavioral aversion with the enhanced transcriptional response seen in our experiments. To test if this was the case, we stimulated 5-HT release using optogenetic methods while simultaneously exposing animals to the odor of the attractive *E. coli* HT115 (figs. S1C and S7C). We predicted that although HT115 does not activate HSF-1 or evoke an avoidance response on its own, optogenetically exciting serotonergic neurons so as to activate HSF-1 (7, 59) in the presence of HT115 odor may change the valence of HT115 from that of attraction to one of aversion. Animals that were stimulated to increase serotonin release while experiencing the odor of HT115 now avoided HT115 when given a choice between HT115 and PA14 (Fig. 7, B and C). This aversion was transient but lasted for as long as 45 min. Control animals that were mock-stimulated by light did not change their behavior and, as expected, were attracted to HT115 and repelled by PA14 (Fig. 7, B and C). Thus, inducing 5-HT release during an olfactory stimulus was sufficient to associate olfactory information regarding odor with HSF-1 activation and trigger an aversive response of *C. elegans* to danger.

DISCUSSION

In summary, our data provide a mechanism whereby 5-HT-dependent learning and HSF-1 activation are coupled to elicit behavioral avoidance and transcription of cytoprotective chaperone genes under threat, thus enhancing the survival of the animal (Fig. 7D). Our data suggest that 5-HT release from neurons needs to be reinforced by HSF-1 activation throughout the animal to interpret a signal as aversive. Conversely, HSF-1 itself is activated by 5-HT release in a multistep process. Whether the cellular relocation of HSF-1 in some way mediates learning, or whether learning produces the HSF-1 relocation is an intriguing question and remains to be answered. In our experiments, we show in some detail the interaction between neurosensory experience and HSF-1 in response to the odorants of the toxic bacterium *Pseudomonas*. However, similar responses could underlie the reaction of *C. elegans* to other stressors. The neuroethological significance of the response of *C. elegans* to 2AA seen in our studies is unknown. Our data suggest that 2AA acts as a kairomone (60), an interspecies chemical messenger that appears to benefit the recipient more than it does the emitter. *C. elegans* is a bacterivore and, like related parasitic nematode species, relies on chemical cues to interpret the hostility or hospitality of its environment. However, the observation that 2AA alone does not elicit an aversive response suggests that 2AA does not act as a danger pheromone. Instead, we speculate that 2AA is akin to what a loud noise may signify to a human—a reason for investigation, to be coupled with an avoidance response if confirmed to be associated with danger. 2AA is also secreted by other known pathogens of *C. elegans* such as *Burkholderia* sp. and predators such as arthropods (61–63), perhaps accounting for the ability of *C. elegans* to detect these dangers and to effectively modulate its behavior and stress responsiveness accordingly.

Our data also suggest that neuronal control over HSF-1–dependent transcription of chaperone genes in *C. elegans* is at least a two-step process. The first step, the reversible and anticipatory change in nuclear localization of HSF-1, which is mediated by neurons and 5-HT, preemptively promotes HSF-1 concentration at nuclear regions near RNA pol II. This could conceivably prepare the chromatin and transcriptional machinery for transcription, were the stressor to materialize. Encounter with the actual stressor then enhances chaperone gene expression, potentially increasing the rate or amount of *hsp* gene transcription (Fig. 7D) (46, 47, 55–58). This subsequent, as-yet unknown signal “confirming” the threat appears to be regulated independently, is required for the actual transcription of *hsp* genes (Fig. 7D), and, we hypothesize, confers the specificity of the transcriptional response to the stressor. The exact mechanism by which 5-HT–dependent learning induces HSF-1 to organize into nuclear bodies and the nature of these structures and the genomic regions with which they are associated (64, 65) remain to be investigated. In *Drosophila* and mammalian cells, a fraction of RNA pol II is held paused at *hsp* loci until HSF-1 binding initiates transcription and the release of pol II into the gene body (46, 48, 57, 66, 67). However, consistent with our data, in these cells too HSF-1 binding alone is not the determining event for the release of pol II pausing, because HSF-1 can bind *hsp70* loci without inducing transcription (68).

The multistep activation of a fundamental cytoprotective response to a threat raises intriguing questions. Given its extraordinarily beneficial roles in conferring stress resistance, why not simply activate HSF-1 in anticipation, even upon the slightest hint of stress? We believe that the answer to this may lie in findings that high chaperone gene expression disrupts basic functions of a cell such as growth, division, and secretory functions and increases susceptibility to transformation (69–71). In fact, it has been shown that the amount of chaperones within cells of a multicellular organism is not maintained in excess, suggesting that excess chaperones are detrimental (72). We therefore hypothesize that for cells within a metazoan, activation of HSF-1 needs to be tightly controlled to occur only upon confirmation of danger, so as to prevent the possible disruption of tissue homeostasis. Organisms survive a range of environmental fluctuations and have evolved to colonize a vast diversity of environmental niches despite the sensitivity of protein-based biological processes to environmental perturbations. We believe that our data begin to address one mechanism through which such adaptation could occur.

MATERIALS AND METHODS

C. elegans strains

The following *C. elegans* strains were used. The following strains were obtained from the Caenorhabditis Genetics Center (CGC): Bristol N2 (wild type), MT15434 *tph-1 (mg280) II*, and AQ2050 *lite-1(ce314); ljlIs102 [tph-1;;ChR2::YFP;unc-122::gfp]*. Generation of AM1061 *unc-119(ed9)III,rmSi1[hsf-1p (4kb)::hsf-1(minigene)::gfp::3' UTR (hsf-1)+Cbrunc-119(+)] II; hsf-1(ok600) I* is described in Tatum *et al.* (7).

Growth conditions of *C. elegans* and bacteria

All strains were grown and maintained at 20°C. Ambient temperature was maintained at 20° to 22°C and carefully monitored throughout the experimental procedures. All animals included in the experiments, unless stated otherwise, were 1-day-old hermaphrodites that were staged as L4 animals 24 to 26 hours before the start of the experiment. Worms were grown and maintained at low densities under standard conditions in standard incubators (20°C), as previously described (7). Specifically, animals were fed with *E. coli* OP50 obtained from the CGC that were seeded onto culture plates 2 days before use, and stock strains were maintained at low densities by passaging 8 or 10 L4s onto nematode growth media (NGM) plates and, 4 days later, picking L4 animals onto fresh plates for experiments. The NGM plates were standardized by pouring 8.9 ml of liquid NGM per plate that yielded plates with an average weight of 13.5 ± 0.2 g. Any plates that varied from these measurements were discarded. The *P. aeruginosa* strain PA14 was obtained from the Yahr Laboratory (University of Iowa), and the *S. marcescens* strain DB11 was obtained from the CGC. Both PA14 and DB11 lawns were kept at 25°C for 2 days before use in experiments.

RNA interference

RNAi experiments were conducted using the standard feeding RNAi method (73–75). Bacterial clones expressing the control (empty vector) construct and the dsRNA targeting most of the *C. elegans* genes were obtained from the Ahringer RNAi library (73) now available through Source Bioscience. (www.sourcebioscience.com/products/life-science-research/clones/rnai-resources/c-elegans-rnai-collection-ahringier/). The RNAi clones used in experiments were sequenced for verification before use. The pL4440 empty vector was used as control RNAi. RNAi-induced knockdown was conducted by feeding animals for 24 hours (*ama-1*) or for over one generation, where second-generation animals were born and raised on RNAi bacterial lawns (*hsf-1*). RNAi-mediated knockdown was confirmed by scoring for known knock-phenotypes of the animals subject to RNAi that have been reported in genome-wide RNAi screens in *C. elegans* (slow and arrested larval growth as well as larval arrest at 27°C for *hsf-1* RNAi and second-generation embryonic lethality in the case of the *ama-1* RNAi). Knockdown was further ascertained using either Western blots (HSF-1) or immunofluorescence (AMA-1) to verify a decrease in protein levels.

Olfactory pre-exposure

Bacterial cultures were grown in Luria broth to OD₆₀₀ (optical density at 600 nm) values of between 1.4 and 1.7, and the variation between cultures within an experiment was kept to ± 0.1 . For pre-exposure to bacterial odors, experiments were carried out in a 25°C incubator, and 750 ml of culture was placed in the lid of a 35 mm $\times$ 10 mm petri dish (catalog no. 10799-192, VWR International), which was then placed in the lid of an inverted standard NGM petri dish (fig. S1A). Because the plates were inverted, animals crawled on OP50 lawns on “top” of the plates, whereas the odorant remained at the “bottom,” undisturbed, and so, at no point, did animals come in contact with the odorant. L4 animals were picked onto these NGM plates on OP50 lawns on the previous day and remained on their respective OP50 lawns during the course of the exposure to odor. We verified that no bacterial spores were transferred via this exposure by conducting the same procedure with an unseeded

NGM plate and observing the plates over the course of the next 2 days for bacterial growth. For “naïve” conditions, the animals were not given any odor before the start of the experiments. When the pre-exposure was to water or 2AA (catalog no. A38207, Sigma-Aldrich), 3 ml of water or 1 mM 2AA (kept at 37°C for 5 min before use) was used in place of the bacterial culture, and experiments were carried out at room temperature ranging from 20° to 22°C. When the pre-exposure was to ethanol or *N*-(3-oxododecanoyl)-L-homoserine (3OC12; catalog no. O9139, Sigma-Aldrich), 3 ml of 0.2% ethanol or 10 μM 3OC12 was used and experiments were carried out at room temperature. For experiments with a recovery condition, after the 30 min of olfaction, the plate containing the liquid odorant was removed and animals were allowed to recover at room temperature for 30 min before harvesting for subsequent experiments.

Bacterial lawn choice assays

L4s were harvested 24 to 26 hours before the start of the behavioral choice experiments. Choice plates were seeded and grown at 25°C for 2 days before use. For PA14 lawns, the duration of growth on the NGM plates before the behavioral assay was particularly important. Younger lawns elicited later avoidance behaviors. Bacterial culture (85 ml; OP50, PA14, HT115, or DB11) grown to OD₆₀₀ values of between 1.3 and 1.6 was used for seeding each lawn. The distance between the lawns was 1 inch. After olfactory training (conducted as described above), 30 1-day-old adult animals were transferred to the middle of the choice plates at a point equidistant from the middle of each lawn, and the behavior of the animals was observed at said frequencies for the next 4 hours at room temperature. The number of animals present on bacterial lawns or off bacterial lawns was recorded, and the experimental bacteria CI was calculated using the following equation:

$$\frac{(\# \text{ of animals on experimental bacteria}) - (\# \text{ of animals on control bacteria})}{\sum [(\# \text{ of animals on experimental bacteria}) + (\# \text{ of animals on control bacteria})]}$$

Animals were considered to be on a lawn as long as they were physically in the lawn, be it on the edge or in the middle of the lawn at the time of observation at a maximum of 4.0× magnification.

Chemotaxis assays

Chemotaxis between water and 10 mM 2AA was carried out at room temperature. Water (5 μl) and 2AA (5 μl) (premixed with 0.5 M sodium azide; catalog no. S2002, Sigma-Aldrich—such that the final concentration of 2AA was 10 mM and that of sodium azide was 0.25 M) were dropped onto an unseeded NGM plate. The two spots were 1.5 inches apart from each other. The spots were air-dried for 5 min, and then the chemotaxis assay was carried out by placing 30 day 1 worms (harvested as L4s the day before) at a point on the plate equidistant from the two spots. Worms were counted as having made their choice only if they were immobile at the time points at which observations were made. CI for 2AA was calculated as:

$$\frac{(\# \text{ of animals on 2AA}) - (\# \text{ of animals on water})}{\sum [(\# \text{ of animals on 2AA}) + (\# \text{ of animals on water})]}$$

Exogenous serotonin (5-HT) treatment

Exogenous serotonin treatment was modified from Jafari *et al.* (42). A serotonin solution (catalog no. 85036, Sigma-Aldrich) in sterile water was dropped onto the surface of OP50 bacterial lawns (such that the lawns were fully covered in serotonin) on NGM plates and dried for ~2 hours at room temperature before use. For confirmation of serotonin uptake using immunofluorescence, serotonin concentrations between 2 and 20 mM were used and day 1 animals were placed onto serotonin-treated plates for between 30 min and 2 hours. For the exogenous serotonin choice assays, a 2 mM serotonin solution was used, and L4s were then picked onto these plates and kept at 20°C for 24 to 26 hours before experimenting with these day 1 adults the next day. Olfactory pre-exposure was carried out using OP50 and PA14 bacterial cultures as described above, followed by choice assays as described above.

PA14 survival assays

Survival assays were carried out on day 1 adult worms that had been harvested as L4s the previous day (50 worms per plate). PA14 killing was performed in liquid bacterial culture in six-well dishes (76). OP50 liquid bacterial culture was used as a control. Both PA14 and OP50 bacteria were grown to an OD₆₀₀ range of 0.8 to 1.0. Olfactory pre-exposure was performed as described above using OP50 and PA14, and immediately after olfaction, worms were picked into liquid bacterial culture in the six-well dishes. Plates were covered loosely to allow for air circulation, kept in a 25°C incubator set to 85 rpm, and scored periodically for survival by visualization under a microscope. Animals were scored as dead if they were not moving in response to gentle swirling of the media and if there was no pharyngeal pumping. At the end of the experiment, death was confirmed by pipetting the animals onto unseeded plates and lack of revival.

Longevity assays

Each experiment was carried out on 50 day 1 adults harvested as L4s the previous day. For longevity with olfaction, animals were pre-exposed to water and 2AA as described above and then transferred onto a new OP50-seeded NGM plate. Animals were transferred every 2 days to avoid starvation, until the point where they were no longer capable of reproduction, typically at day 9. The 2AA liquid and water were refreshed on a daily basis. For longevity with ingestion, water and 1 mM 2AA were dropped onto OP50 lawns on NGM plates and allowed to dry for 2 hours before use. Animals were transferred every 2 days until day 9, and the water- and 2AA-treated plates were made fresh on the day of use. Animals were scored as dead if they were not moving in response to tapping of the plate or a gentle touch on the NGM adjacent to the animal. Animals that died of internal hatching were discarded.

Thermotolerance assays

These assays were carried out on day 1 adult worms that had been harvested as L4s the previous day, with 20 worms per plate. Olfactory pre-exposure was performed as described above, and immediately after olfaction, worms were subjected to an extended heat treatment (45 min) in a circulating water bath preheated to 37.5°C. After this heat exposure, the animals were allowed to recover for 16 hours at 20°C and were then scored as live or dead

the following day. The lack of pharyngeal pumping and lack of response to gentle and harsh touch were the criteria used for scoring an animal as dead.

RNA extraction and qRT-PCR

Samples for RNA were day 1 adult worms that had been harvested as L4s the previous day, with 30 worms per plate. Olfactory pre-exposure was performed as described above, and animals were either immediately harvested (olfaction only) or subjected to a PA14 lawn for 10 min (olfaction + lawn) and then harvested. RNA extraction was conducted according to previously published methods (6, 7). RNA samples were harvested in 50 μ l of Trizol (catalog no. 400753, Life Technologies) and snap-frozen immediately in liquid nitrogen. The following steps were carried out immediately after snap-freezing or samples were stored at -80°C . Samples were thawed on ice and 200 μ l of Trizol was added, followed by brief vortexing at room temperature. Samples were then vortexed at 4°C for at least 45 min to lyse worms completely. RNA was then purified as detailed in the manufacturer's protocol with appropriate volumes of reagents modified to 250 μ l of Trizol. RNA pellet was dissolved in 17 μ l of ribonuclease (RNase)-free water. RNA was treated with deoxyribonuclease using the TURBO DNA-free Kit (catalog no. AM1907, Life Technologies) as per the manufacturer's protocol. Complementary DNA (cDNA) was generated by using the iScript cDNA Synthesis Kit (catalog no. 170-8891, Bio-Rad). RT-PCR was performed using LightCycler 480 SYBR Green I Master Mix (catalog no. 04887352001, Roche), in LightCycler 480 (Roche) at a 10- μ l sample volume, in a 96-well white plate (catalog no. 04729692001, Roche). The relative amounts of *hsp* mRNA were determined using the C_t method for quantitation. *act-1*, *syp-1*, and/or *pmp-3* mRNA was used as internal controls. The use of *syp-1*, which is a germ line-expressed gene, controlled for the variable numbers of embryos that were in the animals when they were prepared for mRNA extraction. All relative changes of *hsp* mRNA were normalized to either that of the wild-type control or the control for each genotype (specified in figure legends). C_t values were obtained in triplicate for each sample (technical triplicates). Each experiment was then repeated a minimum of three times. For qPCR reactions, the amplification of a single product with no primer dimers was confirmed by melt-curve analysis performed at the end of the reaction. No-reverse transcriptase controls were included to exclude any possible genomic DNA amplification. Primers were designed using Roche's Universal Probe Library Assay Design Center software and generated by Integrated DNA Technologies. The primers used for the PCR analysis are shown in table S5.

Single-molecule fluorescence in situ hybridization

smFISH probes were designed against F44E5.4/5 by using the Stellaris FISH Probe Designer (Biosearch Technologies Inc.) available online at www.biosearchtech.com/stellarisdesigner. The fixed worms were hybridized with the F44E5.4/5 Stellaris FISH Probe set labeled with Cy5 dye (Biosearch Technologies Inc.), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Ten to twenty 1-day-old adult wild-type or *tph-1(mg280)II* worms per condition (30' OP50 olfaction, 30' PA14 olfaction, 30' OP50 olfaction + 10' PA14 lawn, and 30' PA14 olfaction + 10' PA14 lawn) were harvested, washed once in $1\times$ RNase-free phosphate-buffered saline (PBS) (catalog no. AM9624, Ambion), fixed in 4% paraformaldehyde, and subsequently washed in

70% ethanol at 4°C for about 24 hours to permeabilize the animals. Samples were washed using Stellaris Wash Buffer A (catalog no. SMF-WA1-60, Biosearch Technologies Inc.), and then the hybridization solution (catalog no. SMF-HB1-10, Biosearch Technologies Inc.) containing the probes was added. The samples were hybridized at 37°C for 16 hours, after which they were washed three times with Wash Buffer A and then incubated for 30 min in Wash Buffer A with DAPI. After DAPI staining, worms were washed with Wash Buffer B (catalog no. SMF-WB1-20, Biosearch Technologies Inc.) and mounted on slides in about 12 µl of Vectashield mounting medium (catalog no. H-1000, Vector Laboratories). Imaging of slides was performed using a Leica TCS SPE Confocal Microscope (Leica) using a 63× oil objective. LAS AF software (Leica) was used to obtain and view z-stacks, and quantification was conducted visually by counting the number of F44E5.4/5 puncta present in nuclei in the head, intestine, and germ line of each individual worm.

Western blotting

For all Western blot analyses, animals were day 1 adults. Acute heat shock was performed by wrapping NGM plates with parafilm and sealing plates in a zippered plastic bag. Plates were submerged in a circulating water bath set to 34°C for 10 min. For protein analysis, 30 day 1 adult worms were collected into 18 µl of 1× PBS (pH 7.4), and then 4× Laemmli sample buffer (catalog no. 1610737, Bio-Rad) supplemented with 10% β-mercaptoethanol was added to each sample before boiling for 30 min. Whole-worm lysates were resolved on 8% SDS-PAGE gels and transferred onto nitrocellulose membrane (catalog no. 1620115, Bio-Rad). Immunoblots were imaged using LI-COR Odyssey Infrared Imaging System (LI-COR Biotechnology). Rabbit anti-HSF1 primary antibody (catalog no. HPA008888, Sigma-Aldrich) was used to detect HSF-1, whereas the mouse anti-α-tubulin primary antibody (AA4.3), developed by C. Walsh, was obtained from the Developmental Studies Hybridoma Bank (DSHB), created by the National Institute of Child Health and Human Development of the National Institutes of Health (NIH), and maintained at the Department of Biology, University of Iowa. The following secondary antibodies were used: Sheep anti-Mouse IgG (H&L) Antibody IRDye 800CW Conjugated (catalog no. 610-631-002, Rockland Immunochemicals) and Alexa Fluor 680 goat anti-rabbit IgG (H + L) (catalog no. A21109, Molecular Probes, Invitrogen). The LI-COR Image Studio software was used to quantify protein levels in different samples, relative to α-tubulin levels. Subsequent analysis of protein levels was calculated relative to wild-type controls. For Phos-tag PAGE analysis, Phos-tag reagent was obtained from Wako Pure Chemicals Industries Ltd. and the protocol was provided here www.wako-chem.co.jp/english/labchem/journals/phos-tag_GB2013/pdf/Phos-tag.pdf. Our experiments used 25 µM final concentration of Phos-tag reagent in a 6% SDS-PAGE gel. Full-length recombinant *C. elegans* HSF-1 protein was a gift of R. Morimoto (Northwestern University). For ethylene glycol bis(succinimidyl succinate) (EGS) cross-linking experiments, whole-worm lysate was prepared by washing worms off the plate in lysis buffer [10 mM Hepes (pH 7.4), 130 mM NaCl, 5 mM KCl, 1 mM EDTA, and 10% glycerol] supplemented with 1 mM dithiothreitol (DTT), 0.2% NP-40, and protease inhibitor cocktail (catalog no. 87785, Thermo Fisher Scientific). Worms were lysed in a Precellys 24 homogenizer (Bertin Corp.) with VK05 beads (Bertin Corp.), and cleared lysate was incubated at room temperature with 0, 0.1, or 0.5 mM EGS (catalog no. 21565, Thermo Fisher Scientific) for 30 min. 4× Laemmli sample buffer supplemented with 10% β-

mercaptoethanol was added and samples were boiled briefly for 5 min to quench reactions. Samples were then resolved on a 6% SDS-PAGE gel, and Western blot analysis for HSF-1 was carried out as described above.

Immunofluorescent staining of whole worms and dissected gonads

Anti-serotonin and anti-HSF1 staining was performed following the protocol developed by the Loer Lab (<http://home.sandiego.edu/~cloer/loerlab/anti5htshort.html>), and modifications were described in full in Tatum *et al.* (7). Briefly, worms were picked into 500 μ l of 1 $\times$ PBS (pH 7.4), spun down quickly, and then fixed in 4% paraformaldehyde (catalog no. 15710, Electron Microscopy Sciences) in 1 $\times$ PBS (pH 7.4) at 4°C for 18 hours. Worms were then incubated in β -mercaptoethanol solution for 18 hours, followed by cuticle digestion using collagenase type IV (catalog no. C5138, Sigma-Aldrich). For serotonin staining, the primary antibody was 1:100 rabbit anti-serotonin as in the study by Tatum *et al.* (7). Primary antibody used was 1:100 rabbit anti-HSF1 antibody (Sigma-Aldrich), whereas the secondary antibody used was 1:100 donkey anti-rabbit Alexa Fluor 647 (catalog no. A-31573, Life Technologies). For staining of NPC proteins, we used 1:100 mouse anti-NPC (55) (Abcam, ab24609) and 1:100 rabbit anti-mouse Alexa Fluor 488 (catalog no. A-11059, Life Technologies). Worms were incubated with DAPI in 0.1% bovine serum albumin (BSA)/1 $\times$ PBS for 30 min at room temperature and then washed and mounted on slides in about 12 ml of Vectashield mounting medium (catalog no. H-1000, Vector Laboratories). Imaging of slides was performed using a Leica TCS SPE Confocal Microscope (Leica) using a 63 $\times$ oil objective. LAS AF software (Leica) was used to obtain and view z-stacks. For gonad dissections to stain for RNA pol II, day 1 adult AM1061 worms were picked into 15 μ l of 1 $\times$ PBS (pH 7.4) on a coverslip, and quickly dissected with a blade (product no. 4-311, Integra Miltex). A charged slide (Superfrost Plus, catalog no. 12-550-15, Thermo Fisher Scientific) was then placed over the coverslip and immediately placed on a pre-chilled freezing block on dry ice for at least 5 min. The coverslip was quickly removed, and the slides were fixed in 100% methanol (–20°C) for 1 min and then fixed in 4% paraformaldehyde, 1 $\times$ PBS (pH 7.4), 80 mM Hepes (pH 7.4), 1.6 mM MgSO₄, and 0.8 mM EDTA for 30 min. After rinsing in 1 $\times$ PBST (PBS with Tween 20), slides were blocked for 1 hour in 1 $\times$ PBST with 1% BSA and then incubated overnight in 1:500 mouse anti-pol II (no. MMS-126R clone 8w16g, BioLegend) (77). The next day, slides were washed and then incubated for 2 hours in 1:1000 donkey anti-mouse Cy3 (code no. 715-165-150, Jackson ImmunoResearch Laboratories) before they were washed and incubated in DAPI in 1 $\times$ PBST and then mounted in 8 μ l of Vectashield and imaged as described above. For quantification of nuclear bodies, the images were merged when co-staining was carried out (HSF-1 and NPC or HSF-1::GFP and pol II), and discrete puncta ranging in size from about 400 to 550 nm were counted first in the HSF-1 channel. We then determined whether these HSF-1 puncta colocalized with the puncta in the other channel. When HSF-1 immunostaining alone was carried out, only the HSF-1 channel was used to quantify nuclear bodies.

Optogenetic activation of serotonergic neurons in choice assays

To make experimental ATR+ plates, a 100 mM ATR (product no. R2500, Sigma-Aldrich) stock dissolved in 100% ethanol was diluted to a final concentration of 0.4 mM into OP50 and 250 μ l was seeded onto a fresh NGM plate. Control (ATR–) plates for experiments were

seeded at the same time with the same OP50 culture but without ATR. Plates were kept in the dark and allowed to dry for a minimum of 10 hours before use. Plates were never used later than 1 day after they were seeded with ATR. The *C. elegans* strain used in this experiment was AQ2050 (*ijIs102; lite-1(ce314)*). L4s were harvested onto ATR+ and ATR– plates, and the experiment was carried out on day 1 adult worms that were transferred in sets of 10 worms per plate onto plates containing 5 ml of either ATR+ or ATR– OP50 lawns. All plates were kept in the dark, and animals were allowed to acclimatize to room temperature (20° to 22°C) for at least 30 min before the start of the optogenetic activation.

For the olfactory training, HT115 and PA14 bacterial cultures were grown to an OD₆₀₀ range of 1.4 to 1.7, and five 10- μ l drops of culture (either HT115 or PA14) were placed around the small bacterial lawn. The animals were then immediately illuminated with blue light for 5 min at a 6.3 $\times$ magnification using an MZ10 F microscope (Leica) connected to an EL6000 light source (Leica) and subsequently transferred onto choice plates containing HT115 and PA14 lawns. ATR– animals were treated similarly. During the process of olfactory training and optogenetic activation, animals that moved away from the central lawn were not used for the subsequent choice assay.

Scoring germline nuclei for HSF-1::GFP activation

Olfaction was carried out as described above (see “Olfactory pre-exposure” section) using water and 2AA. The *C. elegans* strain used was AM1061 (*rmSi1 II; hsf-1(ok600) I*). Whole-worm live imaging was carried out using a Zeiss Observer A1 inverted microscope, and animals were immobilized in 25 mM levamisole on 2% agarose pads with coverslips. Nuclei were scored for induction within 10 min after olfaction. Induction was assessed on the basis of the presence or absence of HSF-1::GFP stress-induced nuclear puncta in the nuclei of germ cells located in the two gonads of *C. elegans*. Analysis of the HSF1::GFP puncta was carried out by counting the number of nuclei showing distinct puncta compared to the total number of nuclei present in a single focal plane. Images also included animals put on OP50 (control) or PA14 for 30 min on PA14 at 20°C.

Statistical analysis and N values

For qRT-PCR data expressed as fold change in mRNA levels relative to control, a linear mixed model analysis for a randomized block design was used to compare the different conditions in each experimental data set. This was done to account for variation between different biological replicates, where treatment response was compared within the experiment. The data for this analysis were the response measure expressed as a ratio of control (for example, OP50 or H₂O odor only). Because the distribution of ratios was usually not normally distributed, the natural log transformation was applied to the data to normalize the data distribution, with the log-transformed values used in the analysis. Means in the log scale were then back-transformed to obtain geometric mean estimates in the original scale. The statistical analysis was performed using MIXED procedure in SAS (version 9.4). For all other data sets and where described (choice assays, smFISH/immunofluorescence/fluorescence quantification, Western blot quantification, and ChIP-qPCR analysis), the parametric Student's *t* test (paired) was conducted to test for significance. For longevity/survival assays, OASIS software available online (<https://>

sbi.postech.ac.kr/oasis2/surv/) was used to calculate mean life span, and the log-rank test was used to calculate statistical significance between the different conditions.

For all data quantified and analyzed, *N* values represent at least three independent repeats conducted to generate the number in each of the represented data points. For data that do not require quantification, the experiments were also conducted three or more than three times, and the data shown are representative data. *N* values for choice assays are the numbers of independent repeats of the choice assay. *N* values for qRT-PCR data are the number of repeats of the mRNA measurements per sample. When assays are conducted to compare the difference between different genotypes, only the number of paired repeats is considered to generate the *N* value. The *N* value in smFISH data, or data regarding HSF-1 nuclear bodies, is the number of nuclei counted per animal (per field of observation) per genotype per treatment condition obtained from two to three independent repeats of the experiment. Only *N* of paired experiments are used when genotypes are compared.

Chromatin immunoprecipitation

Preparation of samples for ChIP was performed by modifying the protocols previously described (51, 78). One hundred wild-type day 1 adult animals per condition were collected from NGM plates, washed with 1× PBS (pH 7.4), and cross-linked with 2% formaldehyde at room temperature for 10 min. Reactions were quenched with 250 mM tris (pH 7.4) at room temperature for 10 min and then washed three times in 1× PBS with protease inhibitor cocktail and snap-frozen in liquid nitrogen. The worm pellet was resuspended in FA buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% sodium deoxycholate], supplemented with protease inhibitor cocktail, lysed using a Precellys 24 homogenizer (Bertin Corp.), and then sonicated in a Vibra-Cell Processor (Sonics & Materials Inc.). Pre-cleared lysate was then incubated overnight with 5 µl of rabbit anti-HSF-1 antibody (Sigma-Aldrich), and immunoprecipitation was performed with Protein A/G Magnetic Beads (catalog no. 88802, Pierce). qPCR analysis of DNA was performed using the reagents described above, and the primer sets *syp-1* and *hsp-70* (F44E5.4) were used to respectively quantify nonspecific and specific binding of gene promoters to HSF-1. The primers used for PCR are shown in table S6. The amplified qPCR products were run on agarose gels to verify that ChIP had resulted in the amplification of the appropriate sized band.

Electrophoretic mobility shift assay

HSE probe sequences were obtained from Silva *et al.* (79), and IR700-labeled oligos were obtained from Integrated DNA Technologies. Worm lysate was prepared by washing worms off in 1× PBS (pH 7.4) and immediately snap-freezing them in liquid nitrogen. Worm pellets were thawed on ice and lysed in a binding buffer [10 mM Hepes (pH 7.4), 130 mM NaCl, 5 mM KCl, 1 mM EDTA, 0.2% NP-40, and 10% glycerol] supplemented with protease inhibitor cocktail and 1 mM DTT. Lysis was carried out using the Precellys 24 homogenizer (Bertin Corp.). EMSA binding reactions [lysate, poly(deoxyinosinic-deoxycytidylic) acid, and labeled IR700-HSE probes] were incubated at room temperature for 30 min, except for heat shock-binding reactions, with or without competition using unlabeled probes, which were performed at 35°C for 30 min. Samples were then run out on a 6% acrylamide gel in

0.5× tris-borate EDTA, imaged using LI-COR Odyssey Infrared Imaging System (LI-COR Biotechnology), and quantified using LI-COR Image Studio software. IR700 HSE (forward), taaattgtagaaggttctagaagatgccaga; IR700 HSE (reverse), tctgcatcttctagaaccttctacaattta.

Motility assays

For motility assays, second-generation RNAi animals were used (refer to “Growth conditions of *C. elegans* and bacteria” section). Animals were harvested as L4s the day before the experiment. Day 1 adults were singled onto a lawn of OP50, and a video of the animals’ movement was captured at 0.8× magnification using a Leica MZ120 camera attached to an upright microscope (Leica KL1500) for about 30 s. Videos were analyzed using ImageJ software to measure the distance traveled by the animal, and from this, the velocity of the worm was calculated. The velocities were then used to calculate the time needed for each animal to travel 1 inch (the distance between bacterial lawns in the choice assay as described above).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Olfactory learning enhances HSF-1 activation

(A) Schematic of olfactory training and subsequent choice assay. Animals were reared on OP50, and naïve animals or animals pre-exposed to PA14 or OP50 odors were given a choice between PA14 lawns and OP50 lawns. The PA14 and OP50 lawns on the choice plate were 1 inch apart and grown as described in Materials and Methods. Animals were placed in the center, equidistant from both lawns, and the number of animals that migrated onto each lawn was tracked over time. A choice index (CI) at each time point scored, over 4 hours was calculated as shown. (B) CI for PA14 of wild-type animals pre-exposed to the odor of either OP50 or PA14 and then offered the choice between OP50 and PA14 lawns. Preference was recorded at the times indicated on the *x axis*. $n = 16$ to 17 experiments of 30 animals per condition. Student's paired *t* test, * $P < 0.05$ and ** $P < 0.01$. (C) Survival of *hsf-1*

knockdown animals on PA14. $n = 3$ experiments of 50 animals per condition. Log-rank test, $P = 0$. See table S1. (D to F) *hsp-70* (F44E5.4/F44E5.5), *hsp-16.2* (Y46H3A.3), and *hsp-16.41* (Y46H3A.2) mRNA abundance measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) upon exposing animals that had been trained on OP50 or PA14 odor to a lawn of PA14. Values were normalized to wild-type animals pre-exposed to OP50 odor. $n = 38$ (D), $n = 12$ (E), and $n = 10$ (F) experiments of 30 animals per condition. Pairwise mean comparison from linear mixed model analysis, $**P < 0.01$ and $***P < 0.001$. Data represent means $\pm$ SEM for (B), (D), (E), and (F). Data in (C) represent total animals across all experiments. n.s., not significant.

Fig. 2. 2AA enhances olfactory avoidance behavior and HSF-1 activation

(A) CI of wild-type animals for PA14 after pre-exposure to the odor of either water or 2AA and then offered the choice between OP50 and PA14 lawns. Preference was recorded at the times indicated on the *x* axis. $n = 10$ experiments of 30 animals per condition. Student's *t* test, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (B) *hsp-70* (F44E5.4/F44E5.5) mRNA abundance measured by qRT-PCR in wild-type animals that were pre-exposed to the odor of water or 2AA and subsequently placed on a PA14 or OP50 lawn. Values are relative to animals pre-exposed to water. $n = 3$ to 21 experiments of 30 animals per condition. Pairwise mean comparison from linear mixed model analysis, *** $P < 0.001$. (C and D) Lifespan

curves of animals (C) continuously exposed to water (control) or 2AA odor, or (D) in physical contact with water-treated (control) or 2AA-treated OP50. $n = 3$ experiments of 50 animals per condition. Log-rank tests indicated no significant differences between experimental groups. (E) Survival of wild-type animals on PA14 after pre-exposure to OP50 or PA14 odor. $n = 8$ experiments of 50 animals per condition. Log-rank test, $P < 0.001$. Also see table S4. Data in (A) and (B) represent means $\pm$ SEM, and data in (C) to (E) represent total animals across all experiments.

Fig. 3. Serotonin is required for HSF-1 activation in response to olfactory learning

(A) CI for PA14 of wild-type and *tph-1(mg280)II* animals pre-exposed to the odor of PA14 before being offered the choice between OP50 and PA14 lawns. Preference was recorded at the times indicated on the *x* axis. *n* = 3 to 4 experiments of 30 animals per condition. Student's two-sample *t* test (unequal variance), **P* < 0.05. (B) CI for PA14 of

tph-1(mg280)II animals pre-exposed to the odor of PA14 compared to the choice indices in *tph-1(mg280)II* animals treated with exogenous 5-HT and pre-exposed to PA14 odor before being offered the choice between OP50 and PA14 lawns. *n* = 4 experiments of 30 animals per condition. Student's paired *t* test, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. (C) *hsp-70* (F44E5.4/F44E5.5) mRNA abundance measured by qRT-PCR upon PA14 exposure in wild-type and *tph-1(mg280)II* animals pre-exposed to the odor of OP50 or PA14. Values are relative to wild-type animals pre-exposed to the odor of OP50. *n* = 9 experiments of 30 animals per condition. Pairwise mean comparison from linear mixed model analysis, ***P* < 0.01 for wild type (odor + PA14 lawn). No significance for *tph-1(mg280)II* (odor + PA14 lawn). (D) *hsp-70* (F44E5.4/F44E5.5) mRNA abundance measured by qRT-PCR after exposure of animals to exogenous 5-HT. Values are relative to control water-treated animals. *n* = 7 experiments of 20 to 30 animals per condition. Student's paired *t* test, **P* < 0.05.

Fig. 4. Serotonin-mediated learning activates HSF-1 throughout the animal

(A to C) smFISH confocal micrographs showing *hsp-70* (F44E5.4/F44E5.5) mRNA and 4', 6-diamidino-2-phenylindole (DAPI) in wild-type and *tph-1(mg280)II* animals pre-exposed to OP50 or PA14 odor and subsequently placed on a PA14 lawn. Images are projected z-stack images of 10- μ m sections across the (A) head, (B) intestine, and (C) germ line. Arrowheads indicate *hsp-70* (F44E5.4/F44E5.5) mRNA foci. Scale bars, 10 μ m. (D to F) Quantification of the number of *hsp-70* (F44E5.4/F44E5.5) foci in projected images. $n = 8$ to 11 animals per tissue per genotype per condition, quantified from two to three independent

experiments. Student's paired t test, $*P < 0.05$ for wild type (OP50 odor + PA14 lawn) compared to (PA14 odor + PA14 lawn). No significance for *tph-1(mg280)II* (OP50 odor + PA14 lawn) compared to (PA14 odor + PA14 lawn). Data represent means $\pm$ SEM.

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Fig. 5. Olfactory learning primes HSF-1 through the formation of HSF-1 nuclear bodies
(A) HSF-1::GFP localization in germline nuclei of control animals at ambient temperature, animals on a PA14 lawn, animals exposed to water or 2AA odor, and animals after 30 min of recovery after exposure to water or 2AA odor. Arrowheads indicate HSF-1 nuclear bodies. Scale bar, 5 mm. (B) Quantification of HSF-1 nuclear bodies under all conditions listed in (A). $n = 33$ to 39 nuclei per animal, 17 to 25 animals per condition, across three to four independent experiments. Student's two-sample t test (unequal variance), $**P < 0.01$ for odor only. No significance was detected for (odor + 30-min recovery). (C) *hsp-70* (F44E5.4/F44E5.5) mRNA abundance measured by qRT-PCR after exposure of animals to the odor of water or 2AA and allowed to recover for 30 min on OP50 lawn before being placed on PA14 lawns. $n = 5$ to 9 experiments of 30 animals per experiment. Pairwise mean comparison from

linear mixed model analysis. No significance was detected. **(D)** Confocal micrographs (individual z-sections) showing HSF-1 localization in germline nuclei of wild-type and *tph-1(mg280)II* animals exposed to water odor or exposed to 2AA odor alone before being placed on a PA14 lawn. Scale bar, 5 mm. **(E)** Quantification of HSF-1 nuclear bodies under all conditions shown in (D). $n = 50$ to 55 nuclei per animal, 8 to 18 animals per condition, across two to three independent experiments. Student's two-sample t test (unequal variance), $*P < 0.05$.

Fig. 6. Olfactory learning primes HSF-1 by increasing its association with RNA pol II
(A) Immunofluorescence confocal micrographs (individual z-sections) showing HSF-1, NPC, and DAPI in germline nuclei of wild-type animals exposed to water odor, 2AA odor, or heat shock. Scale bar, 5 mm. **(B)** Quantification of numbers of total HSF-1 nuclear bodies per nucleus and **(C)** HSF-1 nuclear bodies per nucleus that colocalize with NPCs. $n = 30$ to 36 nuclei per animal, four to six animals per condition per experiment, two independent experiments. **(B)** Student's two-sample t test (unequal variance), $*P < 0.05$ when water odor is compared to heat shock. **(C)** No significance between all conditions. **(D)**

Immunofluorescence confocal micrographs (individual z-sections) of HSF-1, RNA pol II, and DAPI in germline nuclei of dissected animals expressing HSF-1::GFP after exposure to water odor, 2AA odor, water odor + PA14 lawn, 2AA odor + PA14 lawn, or heat shock. Scale bar, 5 μm . **(E and F)** Quantification of numbers of (E) total HSF-1::GFP nuclear bodies per nucleus and (F) HSF-1 nuclear bodies per nucleus that colocalize with RNA pol II in (D). n = number of nuclear bodies per nucleus in 68 to 82 nuclei per animal, 8 to 12 animals per condition per experiment, three independent experiments. Student's two-sample t test (unequal variance), $*P < 0.05$. (B, D, and F). Data represent means $\pm$ SEM.

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Fig. 7. 5-HT signaling couples olfactory information with HSF-1 activation to mark sensory stimuli as threats

(A) CI for PA14 of wild-type animals subjected to control RNAi (empty vector) or *hsf-1* RNAi after being exposed to the odor of PA14 or to the odor of the control RNAi-expressing bacteria. Preference was recorded 4 hours after animals were exposed to the corresponding odors and placed on the choice plates. $n = 5$ to 6 experiments of 30 animals per condition. Student's two-sample t test (unequal variance), $*P < 0.05$. Data represent means $\pm$ SEM. (B) Schematic of olfactory pre-exposure to HT115 odor in conjunction with optogenetic excitation of serotonergic neurons followed by behavioral choice assay. ATR+ indicates the presence of all-*trans*-retinal (ATR), which is required for the light-induced excitation of channelrhodopsin in the serotonergic neurons and subsequent release of 5-HT. ATR- indicates control, mock-excited animals. (C) CI for HT115 in animals $\pm$ ATR after optogenetic excitation of serotonergic neurons. The choice offered was between HT115 and

PA14 lawns. $n = 6$ experiments in triplicate of 10 animals per condition. Student's paired t test, $*P < 0.05$. Data represent means $\pm$ SEM. **(D)** Model: 5-HT-dependent olfactory learning facilitates the association between RNA pol II and HSF-1, resulting in enhanced avoidance behavior as well as enhanced transcription of HSF-1 targets in a stressor-specific manner.

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